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Development of a High-Throughput Assay for Two Inositol-Specific Phospholipase Cs Using a Scintillation Proximity Format

Michael E. Bembenek, Sadhana Jain, Andrea Prack, Ping Li, Linda Chee, Wei Cao, Heidi Spurling, Rebecca Roy, Susan Fish, Mihail Rokas, Thomas Parsons, and Rachel Meyers*

Abstract: Inositol-specific PLCs comprise a family of enzymes that utilize phosphoinositide substrates, *e.g.*, PIP_2, to generate intracellular second messengers for the regulation of cellular responses. In the past, monitoring this reaction has been difficult due to the need for radiolabeled substrates, separation of the reaction products by organic-phase extraction, and finally radiometric measurements of the segregated products. In this report, we have studied the enzymatic characteristics of two novel PLCs that were derived from functional genomic analyses using a phospholipid-modified solid scintillating support. This method allows for the hydrophobic capture of the [^{3}H]phosphoinositide substrate on a well defined scintillation surface and the homogenous measurement of the enzymatic hydrolysis of the substrate by proximity effects. Our results show that the assay format is robust and well suited for this class of lipid-metabolizing enzymes.

Introduction

INOSITOL-SPECIFIC PLCs DEGRADE phosphoinositides to liberate diacylglycerol and inositol phosphates (Fig. 1).[1] The hydrolytic products of the enzymatic reaction are important intracellular secondary messengers that stimulate protein kinase C and promote intraorganellar calcium release, respectively.[2,3] The activation of these intracellular downstream cascades helps regulate responses to external stimuli. Isoenzymes of the PLC class are designated by Greek letters into subfamilies—PLCβ, PLCγ, PLCδ, PLCϵ, and PLCζ—and are further subdivided into numeric subclasses. In general, small-molecular-weight activators of the PLCs have been described and include Ca^{2+} as well as phospholipids.[4] The isozymes also show activation by a variety of cellular protein factors. For example, PLCβs and PLCCϵ are coupled to activation of either the α subunits of G proteins (Gq family) or through the $\beta\gamma$ subunits.[5–7] PLCγ is regulated by activation of nonreceptor or receptor protein tyrosine kinases.[8] Upstream activators of the PLCδ family remain undetermined, whereas PLCϵ appears to be regulated, in part, by association with Ras.[9,10] PLCζ seems to be developmentally linked as a sperm-specific phospholipase that promotes calcium oscillations during mammalian embryogenesis.[11]

The PLC isozymes have been implicated in both cardiovascular and neuronal disease states.[12–14] As a consequence, we have identified two human PLCs utilizing transcriptional profiling methodologies (unpublished observations). Both are closely related family members of the PL-Cδ subclass. The first PLCδ, designated as PLC-16835, is expressed significantly in isolated human vessels and may be responsible for a large portion of the total PLCδ activity in blood vessels.[15] The other PLCδ, designated PLC-32544, has been demonstrated to be up-regulated within neuronal tissue upon bradykinin stimulation and may aid in the lengthening neuronal potentiation.[16]

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ABBREVIATIONS: BSA, bovine serum albumin; CPM, counts per minute; CTH, C-terminal 6 × His-tagged; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; NTH, N-terminal 6 × His-tagged; PCR, polymerase chain reaction; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C.

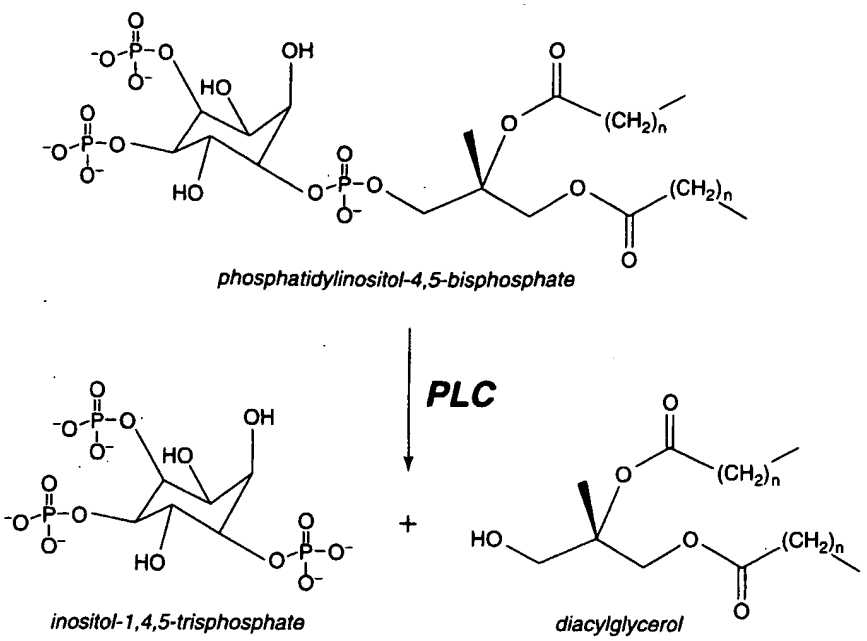

FIG. 1. Reaction scheme of inositol-specific PLC.

Activity assessment of inostiol-specific PLCs usually entails the use of mixed micelles of detergent-solubilized phospholipids containing low amounts of [*myo*-inositol-2-^{3}H(N)]phosphatidylinositol-4,5-bisphosphate (PIP_2). Reaction products require separation, usually by organic-phase extraction, with [*myo*-inositol-2-^{3}H(N)]inositol-1,4,5-trisphosphate remaining in the aqueous phase and diacylglycerol partitioning into chloroform.[17] Until recently, no robust high throughput screening method for this class of enzymes has been available. A novel solid-surface method has been developed that takes advantage of the processivity of PLC. The method has proven to be useful for developing a screening tool of the activity of these two human inositol-specific PLCs. Our efforts were focused on the development of a homogeneous high throughput screening method for these novel enzyme targets. We describe a rapid screening protocol for these novel inositol specific PLCs. utilizing a lipid capture method for phosphoinositides.[18]

Materials and Methods

Cloning and expression of inositol-specific PLCs

Full-length PLC-16835 was pieced together from a partial human keratinocyte cDNA clone and a partial human genomic DNA clone. In brief, the 3′ portion of PLC-16835 (N-terminal 138–3,100) was PCR amplified from a human keratinocyte clone using a 3′ gene specific primer and a 5′ primer with an appended KpnI site.[15] The product was digested with SmaI (5′) and KpnI (3′) (New England Biolabs, Inc., Beverly, MA, U.S.A.). The 5′ end (N-terminal 1–138) was PCR-amplified from human genomic DNA using a 5′ primer with an appended SpeI site and a 3′ gene-specific primer and then digested with SpeI (5′) and SmaI (3′). These two fragments were subcloned into SpeI/KpnI-digested pFASTbac to yield the full-length PLC-16835.

Full-length PLC-32544 was pieced together from cDNA clones derived from a human lung library and a human thyroid library.[16,19] In brief, to generate the full-length, N-terminal 1–878 sequence of PLC-32544, a human lung cDNA clone was PCR-amplified using a 5′ primer with an appended EcoRI site and a 3′ gene-specific primer (New England Biolabs, Inc.). The product was then digested with EcoRI (5′) and BssHII (3′) prior to subcloning. The remaining N-terminal 878–1,284 sequence was cloned from a human lung cDNA library with 5′ and 3′ gene-specific primers. The PCR products were digested with BssHII (5′) and XhoI (3′). These two fragments were subcloned into EcoRI/XhoI-digested pFASTbac to generate a partial PLC-32544 cDNA (N-terminal 1–1,284). The N-terminal 1,284–2,186 sequence was PCR-amplified from the same human lung clone as above, using gene-specific primers and digested with XhoI (5′) and HindIII (3′). Finally, the N-terminal 2,186–4,088 sequence was amplified from a human thyroid cDNA clone using gene-specific primers and digested with HindIII (5′) and KpnI (3′). These two fragments were subcloned into the above pFASTbac partial clone and used to generate the full-length PLC-32544 (Invitrogen™, Life Technologies, Inc., Carlsbad, CA, U.S.A.).[16]

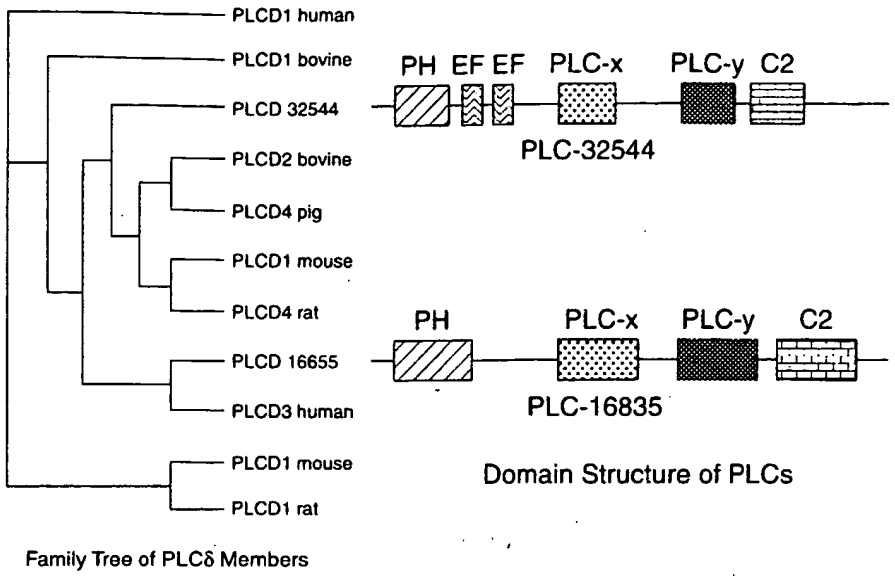

FIG. 2. Comparison of the PLCδ family members with PLC-16835 and PLC-32544 and domain structural analysis. Family tree was developed from sequence analysis with alignments of closely related family members juxtaposed. Domain analysis was performed with Pfam and illustrates pleckstrin homology (PH) domain, PLC-x, -y, and C2 forming the catalytic domains, and EF hands hinge region.[3]

Sequence alignments of each PLC were performed and closely related PLCδ family members were aligned on a family tree and the domain comparison obtained from Pfam analysis (Fig. 2). PLC-16835 was most closely related to PLCδ3 and may represent a splice variant form of the enzyme—showing an extended N-terminus (5′ end). PLC-32544, on the other hand, appears to be a unique member of the PLCδ family, being most closely related to the PLCδ4 subclass. Analysis of the sequence by Pfam demonstrated the presence of the pleckstrin ho-

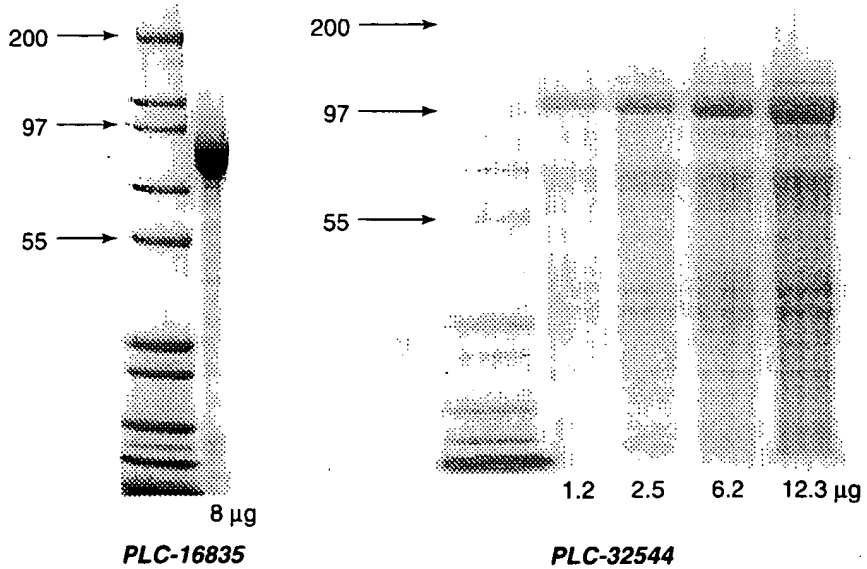

FIG. 3. Analysis of purified recombinantly expressed PLC-16835 and PLC-32544. Purified samples of PLC were denatured under reducing conditions in sodium dodecyl sulfate and analyzed by polyacrylamide gel (10% cross linker) electrophoresis using a Novex gel system (Invitrogen). Gels were stained with Coomassie Blue Reagent, and molecular mass standards, identified in the left lane with arrows, were used as reference markers shown as kDa.

mology domains and the catalytic domains (PLC-x, -y, and C2) for each enzyme and the addition of a pair of EF-hinge regions for PLC-32544.[3]

Baculovirus expression of PLCδs, 16835 and 32544, was achieved for the CTH PLC-16835 and NTH PLC-32544. Clonal expression of each was generated using the Bac-to-Bac™ Baculovirus expression system (Invitrogen™, Life Technologies, Inc.). In brief, the coding sequence for the full-length PLC-32544 was cloned into the pFastBac HTa transfer vector to generate the NTH construct. The open reading frame for the full-length PLC-16835 was cloned into a modified pFastBac vector that allowed for the CTH fusion. Each of the two His-tagged fusion constructs was verified through DNA sequencing analysis. The recombinant transfer vectors were transformed into DH10Bac and plated onto Luria agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal, and 40 μg/ml isopropyl β-D-thiogalactopyranoside (Sigma–Aldrich, St. Louis, MO, U.S.A.). White colonies were selected for inoculation and isolation of recombinant bacmid. Recombinant bacmid was verified by PCR amplification with the pUC/M13 primers. The recombinant bacmid was used for transfecting Sf9 insect cells using CellFECTIN® to generate baculovirus. At 5 days post transfection, recombinant baculovirus was harvested and the cells collected. Cell extract was prepared in lysis buffer containing 50 m*M* sodium phosphate, pH 8.0, 300 m*M* NaCl, and 1% Tween 20 (Sigma–Aldrich), and total cell extract and soluble fraction were analyzed by western blot analysis using poly-His antibodies (Pierce, Rockford, IL, U.S.A.) for verification of PLC expression.

Protein purification

Typically, transfected Sf9 insect cells were grown in 10-L bioreactors for between 48 and 72 h. Cells were harvested from 20 L and the cell paste (~500 g) resuspended in a lysis buffer of 40 m*M* Tris, pH 7.2, 0.4 *M* NaCl, 5 m*M* 2-mercaptoethanol, 10% (vol/vol) glycerol, 1 m*M* phenylmethylsulfonyl fluoride (Sigma–Aldrich), and Complete-EDTA Free™ protease cocktail (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) at 4°C. All further purification steps were conducted at 0–4°C. Cells were homogenized in a Microfluidizer™ (Fluid Dynamics, Newton, MA, U.S.A.), and after a low-speed centrifugation step, the crude cell lysate, ~1,000 mg of total protein, was batch bound to Ni^{2+}-NTA agarose resin (Qiagen, Valencia, CA, U.S.A.). The resin was packed into a column and rinsed exhaustively with 20 m*M* imidazole, pH 7.2, in lysis buffer to remove loosely bound protein. Bound protein was eluted with 250 m*M* imidazole in lysis buffer, pooled, and dialyzed versus 40 m*M* Tris, pH 8.0, 0.4 *M* NaCl, 5 m*M* 2-mercaptoethanol, 10% glycerol. Finally, samples were diluted 1:3 with 20 m*M* HEPES, pH 7.2, 1 m*M* DTT (Sigma–Aldrich), and batch bound to heparin-Sepharose CL-6B resin (Amersham Biosciences, Piscataway, NJ, U.S.A.); the resin was collected by a brief centrifugation and packed into an FPLC column. Specifically bound protein was eluted with a linear salt gradient at ~600 m*M* NaCl, 20 m*M* HEPES, pH 7.2, 1 m*M* DTT. Protein eluates, overall yield of ~3 mg/L Sf9 cells, were diluted with glycerol to 50% (vol/vol) and stored at −20°C. Purity of each of the recombinantly expressed full-length His-tagged PLCs was analyzed by polyacrylamide analysis as illustrated in Fig. 3.

Activity determination of recombinantly expressed PLCs

Phospholipid FlashPlates™ (96-well; PerkinElmer, Inc., Wellesley, MA, U.S.A.) were coated at 0.2 ml/well with either [^{3}H] PI or [^{3}H] PIP_2 (specific activities ~60 Ci/mmol, PerkinElmer, Inc.) at concentrations between 0.5 and 1 μCi/ml (~40–80 n*M*) in Phospholipid Coating Buffer according to the manufacturer's guidelines. In brief, Phospholipid FlashPlate Coating Buffer 10× Concentrate was diluted in deionized water to a final concentration of 1× and the radiotracer added directly to the vessel followed immediately by vigorous mixing. In most cases, radiotracer solutions were allowed to coat the phos-

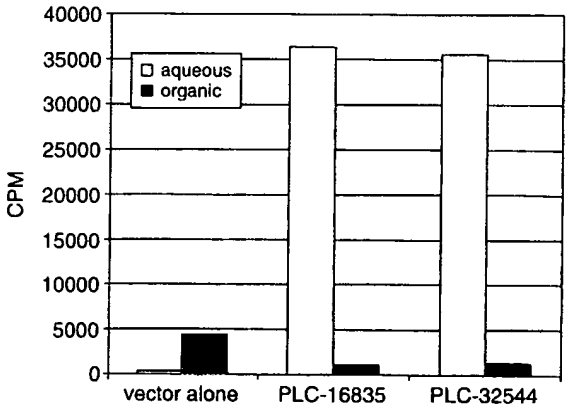

FIG. 4. Distribution of ^{3}H following organic extraction of products released into reaction wells by PLC of [^{3}H]PI-coated phospholipid FlashPlates. Reactions were carried out in 0.2 ml of HEPES-Tris, pH 7.5, 0.1 *M* NaCl, 0.11 m*M* $CaCl_2$, 0.1 m*M* EGTA, 2 m*M* DTT, 1 m*M* sodium deoxycholate of a precoated 96-well phospholipid FlashPlate with ~1 μg of crude protein lysate/well for vectors with recombinant PLC and 10 μg of protein lysate/well of vector alone ([^{3}H] PI initial CPM ~16,000). The reaction was ~75% complete at 1 h at room temperature. Individual well contents were removed and extracted with 2 volumes of $CHCl_3/CH_3OH/HCl_{conc}$ (2:1:0.01, vol/vol) and the phases separated by brief centrifugation. Aliquots of the organic (solid columns) and aqueous layers (open columns) were removed and dried to remove residual $CHCl_3$. Scintillation fluid was added and then samples radioassayed. CPM are normalized for volume differences between the two phases.

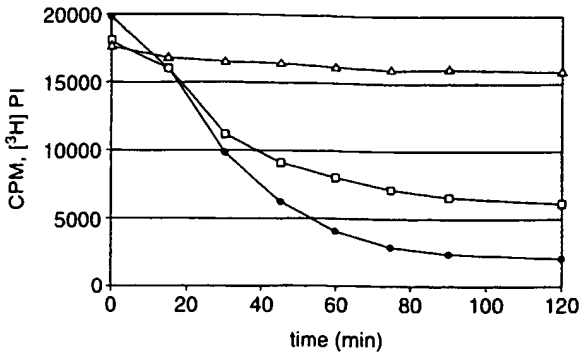

FIG. 5. Comparison of the hydrolysis of [^{3}H] PI from phospholipid FlashPlate with PLC-16835 and PLC-32544. Reactions were carried out at room temperature in 0.15 ml of 50 mM HEPES-Tris, pH 7.3, 0.15 *M* NaCl, 2 m*M* DTT, 0.1 m*M* EGTA, 0.11 m*M* $CaCl_2$, 0.01% acetylated BSA, and 1 m*M* sodium deoxycholate (for PLC-16835 and buffer blanks only) on a precoated [^{3}H]PI phospholipid FlashPlate. Duplicate reactions were initiated with the addition of either 50 μl of buffer alone (Δ), 10 ng of PLC-16835 (in 50 μl; □), or 10 ng of PLC-32544 (in 50 μl; ◆) and the CPM monitored on a PerkinElmer TopCount over the times indicated. Standard errors were no more than 10% for each set of duplicate measurements.

pholipid FlashPlates until the count rate stabilized to a reproducible and fixed measurement using a PerkinElmer TopCount™ instrument (usually between 15,000 and 20,000 CPM). In some instances, the plates were then washed to remove unbound radiotracer and stored at 4°C in 50 m*M* HEPES-Tris, pH 7.5, 0.1 *M* NaCl, 0.02% NaN_3. Plates were stored in this manner for longer than 6 weeks with no effect upon overall performance.

Reactions were carried out typically at room temperature in a final volume of 0.2 ml of 50 m*M* HEPES-Tris, pH 7.5, 0.1 *M* NaCl, 0.2 m*M* $CaCl_2$, 0.1 m*M* EGTA, 2 m*M* DTT, 1 m*M* sodium deoxycholate (for PLC-16835 only), 0.01% acetylated BSA (Sigma–Aldrich). In assays varying the relative Ca^{2+}_{free} concentrations, calcium chloride was titrated with EGTA according to previously described protocols to achieve the final effective concentration.[20,21] Progress of the reactions was monitored on a PerkinElmer TopCount instrument. All experimental results shown in the figures are representative of experiments repeated at least three separate times in either duplicate or triplicate determinations with standard errors below 10% of the mean value.

Results and Discussion

Initial experiments confirmed the correlation between the loss of radioactivity (bound [^{3}H]PI) from the phospholipid FlashPlate surface as directly related to hydrolysis of the phosphoinositide headgroup by recombinant PLC-16835 and PLC-32544 (Fig. 4). As shown, the release of radioactivity from the surface of the phospholipid FlashPlate resulted in the production of only aqueous soluble, *i.e.*, [^{3}H] inositol-1-phosphate, radioactivity following acidic chloroform/methanol extraction with recombinant PLC (compare with vector control). It should be noted that vector alone showed a low, but significant, displacement of bound [^{3}H] PI when compared with recombinant PLC. However, the release was probably due to the 10-fold excess of crude protein (including lipids) in the vector control sample and probably represents the competitive displacement of radiolabeled lipid from the surface. The hydrolysis of substrates by both PLC-16835

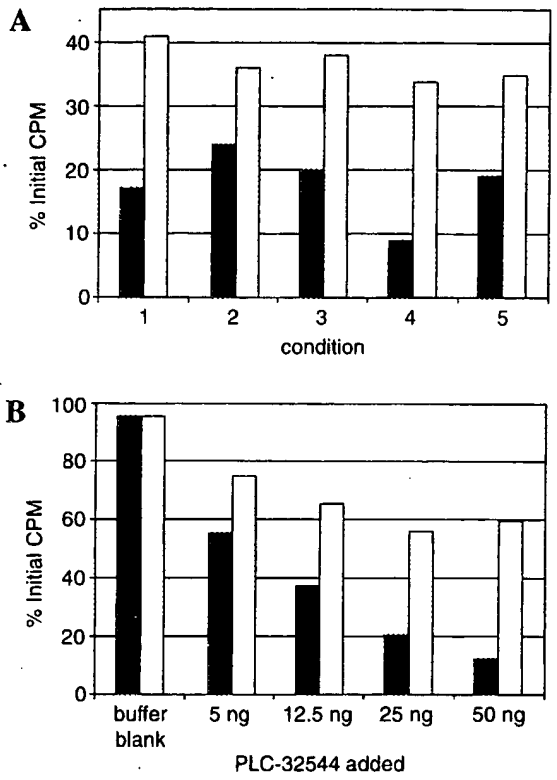

FIG. 6. Comparison of the hydrolysis of [^{3}H]PI (solid columns) or [^{3}H]PIP_2 (open columns) from phospholipid FlashPlate with PLC-32544. Duplicate reactions were carried out at room temperature in a final volume of 0.2 ml of 50 mM HEPES-Tris, pH 7.3, 0.15 *M* NaCl, 2 m*M* DTT, 0.1 m*M* EGTA, 0.2 m*M* $CaCl_2$, 0.01% acetylated BSA. (**A**) Precoated [^{3}H]PI or [^{3}H]PIP_2 (alone, columns in 1) or [^{3}H]PI or [^{3}H]PIP_2 co-coated in the presence of 10 μg/ml sonicated phosphatidylethanol (columns in 2), phosphatidylserine (columns in 3), phosphatidylcholine (columns in 4), or a mixture of all three phospholipids (columns in 5) on a phospholipid FlashPlate. Reactions were initiated with the addition of 10 ng of PLC-32544 (in 50 μl of reaction buffer) and the CPM monitored both initially and after a 2-h incubation. (**B**) Ability of various amounts of PLC-32544 to hydrolyze PI or PIP_2 following a 3.5-h incubation. Data are normalized for the % of CPM released after the initial measurement at time zero with standard errors varying no more than 10%.

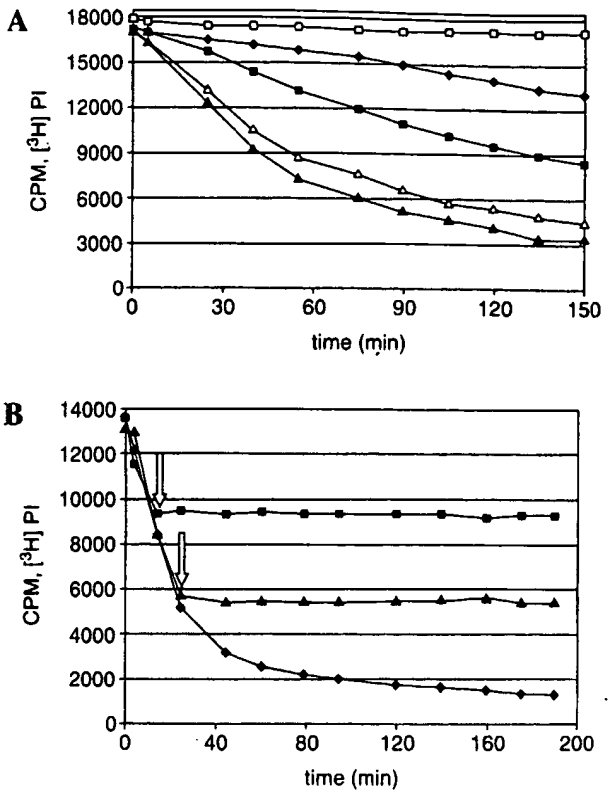

FIG. 7. Dependence of hydrolysis of [^{3}H]PI upon concentration of Ca^{2+}_{free}. Reactions were carried out on a precoated [^{3}H]PI phospholipid FlashPlate at room temperature in a final volume of 0.2 ml of 50 m*M* HEPES-Tris, pH 7.3, 0.15 *M* NaCl, 2 mM DTT, 0.01% acetylated BSA, 0.1 m*M* EGTA, at various free concentrations of Ca^{2+} (**A**) or 0.2 m*M* $CaCl_2$ (**B**). Reactions were initiated in duplicate wells with the addition of either buffer alone or 50 ng of PLC-32544 (A) or 25 ng of PLC-32544 (B) and the CPM monitored on a PerkinElmer TopCount over the times indicated. Panel A represents a titration of calcium according to the methods described previously[20,21] at 5 (◆), 50 (■), 100 (Δ), or 1,000 (▲) μM Ca^{2+}_{free} or buffer alone (□). Panel B represents conditions in which the PLC reaction was stopped without (◆) or with 5 m*M* EGTA, indicated with arrows, at 10 min (■) or 30 min (▲) after the addition of PLC-32544. Standard errors varied no more than 10% for each set of measurements.

and PLC-32544 displayed similar results with either radiolabeled PI or PIP_2 (data not shown) and is in agreement with previously published studies.[18]

A comparison of the reactions upon addition of either recombinantly expressed PLC-16835 or PLC-32544 was performed next. As can be seen in Fig. 5, the loss of radioactivity from the surface of the phospholipid FlashPlate was dependent upon added PLC, as no loss of CPM was seen with buffer alone. These results show that the lipase activity can release up to 90% of the bound counts (PLC-32544), and both enzymes effectively hydrolyze PI from the surface. Furthermore, the progress curves indicate a nonlinear release of CPM indicative of processivity of the enzymes on the surface—typical of nonlinear phase kinetics.[22,23]

Next a comparison was made using the two phosphoinositides, [^{3}H]PI and [^{3}H]PIP_2, as substrates for the reaction (Fig. 6). Based upon previous studies that have demonstrated that added phospholipids can improve PLC activity,[4,24] phospholipid FlashPlates were coated with the radiotracer alone (condition 1) or as mixed micelles containing either phosphatidylethanolamine (condition 2), phosphatidylserine (condition 3), phosphatidylcholine (condition 4), or a combination of all three phospholipids (condition 5, Fig. 6A). PLC-32544 preferred hydrolysis of PI more than PIP_2 under all conditions tested. On average, PIP_2 hydrolysis was roughly 65% complete, whereas PI hydrolysis was slightly better than 80%. Addition of the various phospholipids to the coating buffer

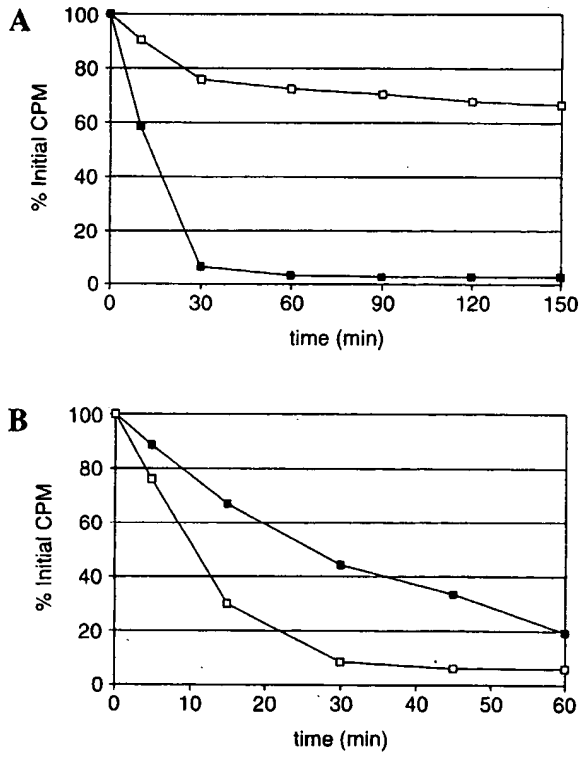

FIG. 8. Effects of deoxycholate upon the hydrolysis of [^{3}H]PI by PLC. Reactions were performed on a precoated [^{3}H]PI phospholipid FlashPlate (~25,000 CPM initially) at room temperature in a final volume of 0.2 ml of 50 m*M* HEPES-Tris, pH 7.3, 0.15 *M* NaCl, 2 m*M* DTT, 0.01% acetylated BSA, 0.1 m*M* EGTA, 0.2 m*M* $CaCl_2$ either with (■) or without (□) 1 m*M* sodium deoxycholate. Panel **A** shows the reaction in the presence of ~300 ng of PLC-16835, and panel **B** shows the reaction with ~100 ng of PLC-32544. CPM monitored on a PerkinElmer TopCount over the times indicated with standard errors varied no more than 10% for each duplicate set of measurements.

did not improve the overall reaction for PIP_2. Hydrolysis of PI, however, reached >90% in the presence of phosphatidylcholine (condition 4). Shown in Fig. 6B, hydrolysis of either radiotracer by various amounts of PLC-32544 was concentration-dependent from 5 ng up to 50 ng, with a preference for [^{3}H]PI over the course of the 3.5-h reaction. Moreover, increasing amounts of PLC-32544 resulted in a significantly greater hydrolysis of PI above 12.5 ng with PI hydrolysis being some five to six times greater than PIP_2 at 50 ng of PLC (~13% versus 60%, respectively). Similar results were obtained with PLC-16835 (data not shown) and, as a result, [^{3}H]PI was used as the substrate for all subsequent analyses.

Previous work had illustrated that the activity of this enzyme class is dependent upon concentrations of Ca^{2+}_{free} and can be stimulated by detergents.[4,24] To define better the activity of these two enzymes using this format, the dependence of the activity upon concentrations of Ca^{2+}_{free} was analyzed (Fig. 7). As demonstrated with PLC-32544, the release of radioactivity from the phospholipid FlashPlate was exquisitely sensitive to various amounts of Ca^{2+}_{free} as titration with EGTA effectively lowered PLC hydrolysis in a concentration-dependent manner from calcium levels of 5 μM to 1 mM (Fig. 7A). Thus, at the lowest concentration of 5 μM Ca^{2+}_{free} only ~25% of PI was hydrolyzed from the surface, whereas at concentrations above 0.1 mM Ca^{2+}_{free} the reaction approached >80% completion during the 150-min incubation. Moreover, PI hydrolysis could be ablated by addition of excess chelator, *i.e.*, 5 mM EGTA, following the initiation of the reaction with PLC-32544 (Fig. 7B). Addition of 5 mM EGTA (denoted at arrows) at 10 min following PLC addition resulted in only 25% of hydrolysis, whereas addition of excess chelator at 30 min terminated the reaction at ~60% completion. In both instances, the reaction was halted completely and the signal stabilized by the addition of excess EGTA. Similar effects were also observed with PLC-16835 with an optimum effective concentration of Ca^{2+}_{free} of ~0.1 mM (data not shown).

By contrast, the effects of the detergent deoxycholate upon the reaction showed significant differences between these two enzymes (Fig. 8). As shown in Fig. 8A, when detergent was added to the buffer, a stimulation of hy-

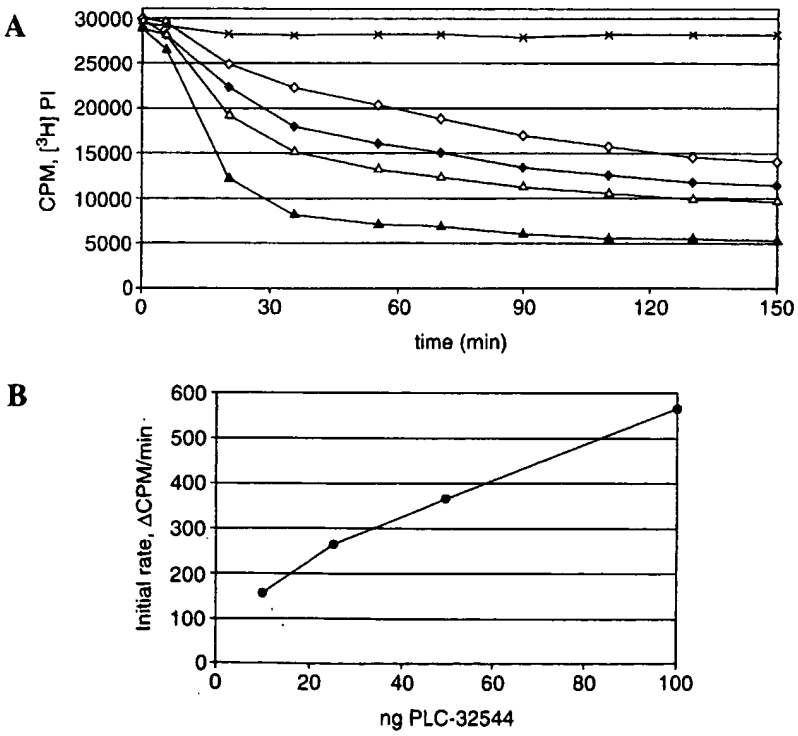

FIG. 9. Dependence upon the hydrolysis of [^{3}H]PI upon various concentrations of PLC-32544. Reactions were performed on a precoated [^{3}H]PI phospholipid FlashPlate at room temperature in a final volume of 0.2 ml of 50 mM HEPES-Tris, pH 7.3, 0.15 M NaCl, 2 mM DTT, 0.01% acetylated BSA, 0.1 mM EGTA, 1 mM $CaCl_2$. Reactions were initiated in triplicate wells with the addition of either buffer alone (×) or 10 ng (◇), 25 ng (◆), 50 ng (Δ), or 100 ng (▲) PLC-32544 (A) and the CPM monitored on a PerkinElmer TopCount over the times indicated. Standard errors varied no more than 10% for each set of measurements. Panel **B** shows a replot of the data measuring the initial reaction rates at 20 min ($CPM_{initial}$ − $CPM_{20\ min}$/20 min) versus concentration of PLC-32544.

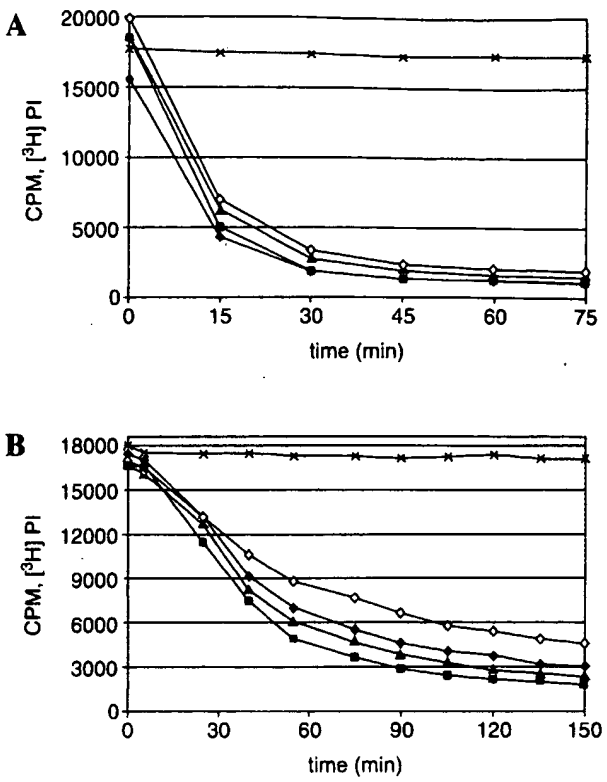

FIG. 10. Effects of DMSO on hydrolysis of [^{3}H]PI from phospholipid FlashPlate with PLC-16835 (**A**) and PLC-32544 (**B**). Duplicate reactions were carried out at room temperature in a final volume of 0.2 ml of 50 m*M* HEPES-Tris, pH 7.3, 0.15 *M* NaCl, 2 m*M* DTT, 0.1 m*M* EGTA, 0.2 m*M* $CaCl_2$, 0.01% acetylated BSA, 1 m*M* deoxycholate (**A** only) at concentrations of DMSO (vol/vol) of 0% (◇), 0.5% (◆), 1% (▲), or 2% (■). Buffer blanks (×) were assayed at 2% DMSO (vol/vol) in the matched PLC buffer. Reactions were initiated with the addition of 50 ng of PLC-16835 (in 50 μl of reaction buffer) or 50 ng of PLC-32544 (in 50 μl of reaction buffer) and the CPM monitored for the times indicated on a Packard TopCount. Standard errors varied no more than 10% for each set of measurements.

drolysis was observed with PLC-16835. In this instance, the reaction was nearly complete at 30 min and only 25% complete in the absence of 1 m*M* deoxycholate. Moreover, in the absence of deoxycholate, PLC-16835 was unable to hydrolyze PI significantly beyond 30 min. On the other hand, PLC-32544 showed marked inhibition of the reaction rate by addition of deoxycholate to the buffer (Fig. B). At 30 min, in the presence of detergent, ~55% of the reaction had proceeded to completion, whereas 95% was achieved in its absence. Although the detergent slowed the reaction rate, PLC-32544 hydrolysis neared completion by 1 h. Interestingly, two other detergents, CHAPS and β-octylglucoside, showed similar effects upon PLC-32544 above one-half the critical micellar concentration of the detergent (data not shown).

As shown in Fig. 9, the hydrolysis of PI was dependent upon the amount of PLC added as represented with PLC-32544. Thus, the reaction extent and rate were maximally achieved at 100 ng of PLC-32544 (~5 n*M*) with ~85% hydrolysis at 150 min (Fig. 9A). Lower concentrations of PLC-32544 displayed proportionately less hydrolysis at the same time, with 10, 25, and 50 ng producing ~50%, ~60%, and ~67% reduction of CPM, respectively. Analysis of the initial reaction rates, within the first 20 min. (Fig. 9B), displayed linear dependence of the hydrolysis upon concentration of PLC-32544.

Tolerance of the PLC reaction to added DMSO in the reaction buffer was tested next (Fig. 10). As can be seen in Fig. 10A, PLC-16835 was unaffected by the total range of concentrations of DMSO from 0.5% up to 2% (vol/vol), displaying similar initial reaction rates and extent of hydrolysis. Interestingly, PLC-32544 showed some sensitivity to DMSO (Fig. 10B). In this instance, DMSO showed a slight enhancement, ~10%, of both reaction rate and the extent of the reaction as the concentration of DMSO increased: 73% hydrolysis in the absence versus ~86% in the presence of DMSO. The solvent effects of DMSO and that seen with detergents at or above one-half critical micelle concentration upon PLC-32544 activity indicate a sensitivity of the enzyme to alterations of either the dielectric constant of the buffer or a direct impact on the enzyme structure/activity directly. This represents an intriguing observation for the PLCδ class (authors' personal observations) and will require further research in order to understand the exact underlying mechanism.

Our results demonstrate the utility of this assay format for monitoring the activity of these novel human inositol-specific PLCs and support our initial observations with bovine PLCδ1.[18] The assay complements a recent report for monitoring the inositol phosphate production following cellular receptor activation.[25] Thus, together these formats should provide important tools for understanding the molecular mechanisms of inhibition following receptor signaling events through the PLC-mediated pathway.

The utility of this phospholipid FlashPlate format provides the basis for a series of other lipid-metabolizing enzymes and has been utilized effectively for both fatty acid synthase and phosphoinositide 3-kinase.[26,27] Owing to the inherent difficulty of assaying this functional class of enzymes, it seems possible that other family members, *e.g.*, diacylglycerol kinase, diacylglycerol acyl-CoA transferases, may find utility for assay development by using this platform.

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